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13. ABSTRACT *(Maximum 200 words)*

This project was focused on further development of our technique to stabilize platelets with light fixation and then freeze-dry for long term storage. The stabilization conditions have been well-established as optimized and now the whole process has been scaled up to produce full transfusion doses for use in animal studies or for other preclinical work in the pursuit of an IND with the FDA. The specific aims were directed at investigating the ability of rehydrated lyophilized platelets (RLP) to mount an activation response, to interact with fresh platelets and thereby boost functionality, to generate a near-normal clot signature in vitro, and to restore hemostasis in swine receiving a "wash-out" with an artifical hemoglobin solution. In addition, the immunogenicity of canine platelet freeze-dried preparations was tested with multiple infusions in recipient animals. The results demonstrated phosphorylation and other activation-related activities in RLP upon stimulation with physiologic agonists, enhanced adhesion to thrombogenic surfaces in the presence of a small number of fresh platelets, and effective hemostatic activity in the Clot Signature Analyzer in vitro and in the washout swine model in vivo, with very good tolerance of repeated RLP infusions. These findings support the notion that RLP should proceed to an FDA application for clinical trials.

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FINAL TECHNICAL REPORT

GRANT# : N00014-97-1-0891

PRINCIPAL INVESTIGATOR: Dr. Arthur P. Bode (ECU) (e-mail: bodea@ecu.edu) and
Dr. Thomas H. Fischer (UNC subcontract)

INSTITUTION: East Carolina University Brody School of Medicine, and
The University of North Carolina at Chapel Hill (subcontract)

GRANT TITLE: Immunogenicity and Stability of Lyophilized Platelets for Transfusion Medicine

AWARD PERIOD: June 15, 1997 to Sept 30, 2001.

OBJECTIVE: To demonstrate the ability of fixed freeze-dried platelets to promote hemostasis in a variety of animal models and *in vitro* conditions, and to examine the extent of immunogenic response elicited by multiple infusions.

APPROACH: This has been a collaborative effort with principal investigators at ECU and UNC-Chapel Hill, using several separate animal models and multiple *in vitro* analysis techniques. At ECU, the primary focus of *in vitro* testing has been on the Clot Signature Analyzer as a test of global hemostatic activity of rehydrated platelets in a milieu of fresh or reconstituted whole blood, and on use of the Baumgartner perfusion system to assess adhesion of rehydrated platelets to physiologic thrombogenic surfaces of injured vessel segments. In addition, much attention has been put into optimizing the process for stabilization of platelets in preparation for freeze-drying to produce a stable material. These preparations were tested also at UNC to define their biochemical responsiveness (human platelets) and immunogenic properties (canine platelets infused in dogs). Porcine platelets prepared by our process were tested at UNC in pilot studies of co-infusion with pigs receiving a trial HBOC solution in a blood volume exchange model that also removed almost all the circulating platelets. Studies on the interaction of our fixed freeze-dried platelets with HBOC solutions or freeze-dried red cell preparations are still in the preliminary stages.

ACCOMPLISHMENTS: Although this project is no longer funded by ONR, it has enough momentum now to be developed into a business plan for commercialization and implementation as a potential new transfusion product. ECU and UNC have jointly started a spin-off business that will pursue this goal to apply for an IND with the FDA and then enter clinical trials of the lyophilized platelets in treatment of acute hemorrhage. The past support of grants from ONR has made this possible and we believe that these final steps in development will culminate in the ultimate goal of creating a deliverable product to the armed services as a measure of success of the past investment. The first human infusion studies with these platelets were intended to be performed in the summer of 2000 with "Plus-up" funding from ONR; this money was given in addition to the core funds for Year 3 of this project and was immediately budgeted for use in a new subcontract written with the American Red Cross Blood Services Laboratory in Norfolk, VA, for autologous infusion of radiolabelled rehydrated platelets in at least four donors. However, because of funding restrictions imposed by the ONR project officer at the time, it was not possible to implement the new Plus-up budget and the core money in the same period, so the planned studies could not be carried out. Even though the original plan could not be carried forward to the first human use of these lyophilized platelet preparations, we are engaged in

bringing their production up to GLP/GMP standards so that infusion studies can be carried out in the future as new resources permit. We now anticipate performing Phase I human trials within 18 months after capitalization of the spin-off company. As the clinical trials proceed, we will endeavor to establish a role for the lyophilized platelets in field trials for combat casualty care and for development of a strategic national blood reserve for military and civilian use.

The rest of this section lists major findings from specific investigations of the current grant.

(1) Stimulus response coupling is retained in the fixed, freeze-dried platelets.

RL (rehydrated, lyophilized) platelets have been shown to retain many native functions of fresh platelets *in vitro* and to mediate hemostasis *in vivo*. The goal of these studies was to determine if RL platelets provide positive feedback amplification in thrombus formation. First, we showed that exposure of RL platelets to thrombin resulted in enhanced phosphorylation of several intracellular proteins, including 18 kDa and 42 kDa kinase substrates which were shown to be the myosin light chain (substrate for myosin light chain kinase) and pleckstrin (substrate for protein kinase C). These results indicate that RL platelets retain some of the activation-dependent intracellular functions of fresh platelets that underlie the activation response. Secondly, we showed that RL platelets express active glycoprotein IIb-IIIa complex, bind fibrinogen and associate with fibrin polymers in thrombus formation and adhesion to subendothelial matrix. Thirdly, we demonstrated that RL platelets undergo an activation-dependent secretion response. Finally, the thrombogenicity of RL platelets, which is in part mediated by activation-dependent surface exposure of phosphatidylserine, increases in an activation-dependent manner. These results indicate that RL platelets, because of their ability to amplify hemostatic reactions, hold promise as a transfusion product for providing immediate hemostasis.

(2) RL platelets mediate normal thrombus formation for hemostasis.

To further evaluate the functional properties of this transfusion product, we studied the role of RL platelets in three aspects of thrombus formation and lysis. First, the interaction between RL platelets and fibrinogen was investigated. After ADP activation, RL platelets bound exogenous ^{125}I-labeled fibrinogen in a dose-dependent manner with an affinity that is similar to that of fresh platelets and was inhibited by RGD peptides. ^{125}I-Labeled fibrinogen binding to RL and fresh platelets respectively saturated at 14,000 and 32,000 molecules per cell. Scanning electron microscopic ultrastructural analysis showed that fibrin strands interacted with the surface of RL platelets in a normal manner. The second set of studies investigated the ability of RL platelets to catalyze and amplify the clot formation process in an activation-dependent manner. We showed that RL platelets undergo degranulation in fibrin clots and functioned as thrombogenic surfaces for the generation of activated coagulation factors and fibrin generation. A final set of studies was performed to investigate fibrinolysis of clots that contained RL platelets. RL platelet clots were lysed in the presence of tissue plasminogen activator with a similar time course as clots without platelets, and lysis occurred faster than when fresh platelets were included in the fibrin mass. The results of these three studies demonstrate that RL platelets are capable of mediating thrombus formation and do not inhibit lysis.

(3) RL platelets provide hemostasis in porcine model for washout (dilutional) thrombocytopenia.

A widely recognized complication from extensive fluid replacement is the induction of coagulopathies related to the "washout" dilution of components of hemostatic systems. As HBOC preparations come into wider use in military medicine, the extensive degrees of exchange (e.g. < 1% initial RBC count) that can be achieved with acellular oxygen carries are expected to result in more severe bleeding disorders, potentially resulting in spontaneous hemorrhage and neurological disorders.

UNC has developed a porcine blood-HBOC-201 exchange model to investigate vascular and hematological path physiological responses to exhaustive exchange with acellular oxygen carriers. Pilot studies were performed in pigs to ascertain if rehydrated, lyophilized porcine platelets were capable of providing hemostasis after extensive exchange with Biopure hemoglobin-based oxygen carrier (HBOC) 201. Pigs were anesthetized, and then several sensors were placed to follow hemodynamic and vasoactive processes. Blood and HBOC-201 were respectively withdrawn and infused from contralateral femoral veins at rates of approximately 40 ml/min while maintaining hemodynamic parameters in the normal range. Red blood cell and platelet levels fell in an approximately exponential manner with rates of 5% per minute ($t_{1/2}$ = 20 min). Bleeding times from ear vein lacerations and 23 gauge needle punch sites in the jugular vein were measured before exchange and during the exchange time course. Ear vein bleeding times increased during the exchange period from initial values of 2 to 3 minutes to >10 minutes at the 60 minute time point, when platelet and RBC levels were less than 15% of initial values. Similarly, jugular vein wound bleeding, which initially ceased in less than 2 minutes, was indefinite (uncontrollable hemorrhage) after RBC and platelet counts were lowered to less than 30% of initial values. Fluorescent-labeled porcine RL platelets, equivalent to 34% of initial platelet mass, were infused over a 5-minute period after 70 minutes of exchange when endogenous platelet levels were less than 15% of initial values. Uncontrolled hemorrhage from the jugular vein wounds *ceased* after the RL platelet infusion and bleeding times from ear vein lacerations shortened to more normal values between 5 to 7 minutes. Analysis of wound sites with fluorescent microscopy demonstrated that RL platelets adhered to damaged vascular tissue to form a platelet-rich, but fibrin-depleted plug. These results indicate that hemostasis can be obtained in dilutional thrombocytopenia when RL platelets are co-infused with HBOC-201.

(4) Infusion of RL platelets does not induce an immune response to endogenous platelets or cause thrombocytopenia.

An additional finding that is important for the development of RL platelets is that multiple infusion of RL platelets (doses equivalent to 6% of the endogenous platelet mass) does not induce antibodies to endogenous platelets or thrombocytopenia. This result indicates RL platelets do not serve as an "adjuvant" for anti-platelet antibodies or do not interfere with thrombopoietin feedback for megakaryocyte maturation and thrombopoiesis. An important aspect of continuation of these studies will be to ascertain if higher doses of RL platelets do not induce anti-platelet antibodies or result in thrombocytopenia.

(5) Addition of RL platelets to thrombocytopenic whole blood restores normal hemostasis in the Clot Signature Analyzer.

The Clot Signature Analyzer (CSA) is a device designed to test platelet function in non-anticoagulated (or reversed) whole blood by simulating an acute hemorrhage under flowing conditions. We have found that RL platelets in preparations produced under our now standardized conditions generate clot signatures in the CSA that are within the range of results expected with whole blood from normal donors. Preparations kept frozen for up to 18 months showed no deterioration of response in the CSA. Those RL preparations that gave less satisfactory performance were found to have reduced levels of GPIb on the platelet surface, usually as a result of over- or under-fixation conditions at the start and degradation over time of storage. In fact, the reliable performance and normality of RL platelets in reconstituted whole blood on the CSA led to an interest of the manufacturer of the CSA, Xylum Corporation of Scarsdale, NY, to pursue studies of the feasibility of using RL platelets as a quality control material for clinical implementation of the CSA. Unfortunately, Xylum discontinued further development of the CSA after delays in submitting a 510K application to the FDA for its use in

diagnostic medicine and has since dissolved. This device is now orphaned but served as a demonstration platform for RL platelet hemostatic efficacy *in vitro*.

(6) RL platelets interact with fresh platelets to boost adhesion in the Baumgartner perfusion test.

It is important to consider that the lyophilized platelets are intended for use mainly in trauma/surgical bleeding patients that will still have a residual number of endogenous functional platelets that can interact with the infused RL platelets. The Baumgartner test is performed by removing all native platelets from fresh donor blood and adding back reconstituted lyophilized platelets before initiating the perfusion. The percent coverage of denuded vessel obtained in this way is typically 20-28% for our standard RL platelets. We have now found out that if we add a small number of fresh platelets (final count = 2-3,000/uL) back to the RL blood sample then the percent coverage of the vessel increased by half or more (to 30 – 45%), suggesting that the modicum of fresh platelets was boosting adhesion of the RL platelets through a recruitment phenomenon. Over-fixed RL platelets did not demonstrate this boost, indicating that responsiveness of the lyophilized platelets was a key factor. The demonstration of a feed-forward interaction between fresh platelets and RL platelets in adhesion parallels what we found in earlier work that showed RL platelets joining fresh platelets in aggregate clumps after addition of ADP or collagen, but not aggregating on their own in the absence of fresh platelets.

(7) Modification of platelet preparation procedure permits upscaling process to produce a full transfusion dose (or more) per run.

In year 3 of this project we purchased a Clean-edge HEPA filter work bench (Baker Corp) using Plus-up funds to begin sterile processing of platelets for lyophilization in anticipation of the first human infusion studies. Although those studies were not carried out (see above), we have scaled up and "cleaned up" the platelet isolation and stabilization techniques to produce unit doses with minimal if any bioburden remaining in the dry powder. Our improved process now combines column chromatography with centrifugation washing steps to minimize open-air exposure and routinely handles two apheresis units at a time (5-6 x 10^{11} plts total). Microbial growth analysis of preps placed in storage is still on-going. GLP-level processing will be necessary for continuation of this project into clinical trials as discussed above. At present we have noted no degradation of RL platelet adhesion function or responsiveness as a result of these processing changes.

SIGNIFICANCE: The results of this work provide an essential knowledge base for the entry of RL platelets into human trials for safety and efficacy.

PUBLICATIONS:

Peer-Reviewed Journals:

1. Bode, A.P., Read, M.S., and Reddick, R.L. (1999); "Activation and Adherence of Lyophilized Human Platelets on Canine Vessel Strips in the Baumgartner Perfusion Chamber." Journal of Laboratory and Clinical Medicine 133: 200-211.

2. Fischer, T., Merricks, E., Russell, K., Raymer, R., White, G., Bode, A., Nichols, T. and Read, M. (2000); "Intracellular Signalling in Rehydrated, Lyophilized Platelets." Brit. J. Haem. 111, 167-175.

3. Bode, A.P., and Read, M.S. (2000); "Lyophilized Platelets: Continued Development." Transfusion Science 22: 99-105.

4. Fischer, T., Merricks, E., Bellinger, D., Hayes, P. Smith, R., Raymer, R., Read, M., Nichols, T. and Bode, A. (2001); "Splenic clearance mechanisms of rehydrated, lyophilized platelets." Artificial Cells Blood Substitutes and Immob. Biotech. 29, 439-451.

5. Bode, A.P., Lust, R.M., Read, M.S., and Fischer, T.H. (2002); "Correction of the Bleeding Time with Lyophilized Platelet Infusions in Dogs on Cardiopulmonary Bypass." J. Lab Clin Med. (in review).

6. Fischer, T., Khandelwal, G., Merricks, S., Raymer, R., Bode, A., Bellinger, D., Russell, K., Reddick, R., Sanders, W., Nichols, T. and Read, M. (2002); "Thrombus Formation and Lysis with Rehydrated, Lyophilized Platelets." Vox Sang. (submitted).

Book Chapter:
7. Bode, A.P. and Read, M.S. (2000); "Lyophilized Platelets for Transfusion Medicine." In Platelet Therapy: Current Status and Future Challenges, J. Seghatchian and E.L. Snyder, eds; Elsevier Science, Amsterdam; pp. 131-167.

Abstracts (Meetings):
1. Bode, A.P., and Read, M.S. (American Association of Blood Banks annual meeting, 1998); "Adhesion of Lyophilized Platelets to Vessel Subendothelium is Glycoprotein Ib - Specific." Transfusion Suppl. 38: 74S.

2. Fischer, H.T., Hickerson, D.H.M., Bode, A.P., and Read, M.S. (Intl. Society of Blood Transfusion annual meeting, 2000) "Intracellular Biochemical Activity in Rehydrated Lyophilized Human Platelets (RL) Under Development as a Transfusion Substitute." Vox Sanguinis 78 (Suppl S1): P185.

3. Bode, A.P., and Bainbridge, M. (Intl. Society of Blood Transfusion annual meeting, 2000); "Use of Freeze-Dried Platelets, Plasma, and Red Cells to Generate a Normal Hemostasis Profile in vitro in the Clot Signature Analyzer (Xylum Corp.)." Vox Sanguinis 78 (Suppl S1): 0036

4. Fischer, T., Merricks, E., Raymer, R., Nichols, T. and Bode, A. (American Society of Hematology annual meeting, 2000); "Multiple infusions of rehydrated, lyophilized platelets does not induce antiplatelet antibodies or thrombocytopenia in a canine model system." Blood 96, 2000.

5. Fischer, T., Merricks, E., Raymer, R., Nichols, T., Hayes, P., Bode, A., Pearce, L. and Manning, J. (American Society of Hematology annual meeting, 2001) The co-infusion of rehydrated lyophilized platelets with HBOC-201 for hemostasis in dilutional thrombocytopenia. Blood 98, 2250.

In addition, data presentations on this project were given by Drs Bode and Fischer at the 2000 and 2001 ATACCC conferences at Ft. Walton Beach, FL.

There were three patents issued on this technology during the term of this grant: (1) Patent No. 5,651,966, awarded July 1997, (2) Patent No. 5,891,393, awarded April 1999, (3) Patent No. 5,901,608, awarded May 1999.